ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1990, p. 1312–1317
0066-4804/90/071312-06$02.00/0

Vol. 34, No. 7

Quantitative Analysis of Antimicrobial Effect Kinetics in an In Vitro Dynamic Model

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Received 29 June 1989/Accepted 4 January 1990

Variants of the available methods for estimating antimicrobial effect kinetics in an in vitro dynamic model were analyzed. Two integral parameters characterizing antimicrobial effect duration (T_E) and intensity (I_E) are suggested to define and analyze the concentration-effect relationships in these models, irrespective of the method of recording. T_E is defined by the time from the moment of antibiotic administration to the moment when the bacterial count again reaches its initial level. I_E is defined by the area between the microbial growth curves in the presence and absence of an antibiotic. T_E and I_E were used to quantify the antimicrobial effects of sisomicin on *Pseudomonas aeruginosa* 58, *Escherichia coli* 93, and *Klebsiella pneumoniae* 5056, simulating the pharmacokinetic profiles of the drugs observed following intramuscular administration in therapeutic doses, including the variability of aminoglycoside concentrations in human blood.

The development of dynamic models that allow the simulation of antibiotic pharmacokinetic profiles for the study of the kinetics of antimicrobial effect (AME) has provided a means for evaluating the role of the pharmacokinetic factor in the development of AME (5, 8, 10) and has stimulated the in vitro assessment of the relative effectiveness of various antibiotics and dosage regimens. However, despite strictly controlled conditions for experiments with dynamic models, conclusions about the preference of one drug or dosage regimen over another are essentially qualitative and not always unequivocal. This results to a considerable degree from deficiencies in the available methods for quantifying AME.

The analysis of AME kinetics or, strictly speaking, the kinetics of microbial growth in the presence of an antibiotic traditionally employs models that describe the initial phase of cell growth inhibition under the action of the drug and compare microbial growth rates in the presence and absence of the antibiotic.

It has been proposed that four parameters be evaluated to characterize more completely the quantitative change in microbial counts in the presence of an antibiotic. These include the time required for microbial count to be reduced by 99% (T_{99}), the time required to reach the minimum count (T_{min}), the minimum bacterial cell concentration (B_{min}), and the time required for a subsequent 10-fold (1-log) increase in bacterial count (t_{1lg}) (19). Unfortunately, the last three parameters can be estimated only by counting CFU after samples are grown on solid nutrient media or with the microcalorimetric technique for T_{99} (4). Since the high sensitivity of colony counts is "compensated" for by its insufficient accuracy, the estimates for the parameters T_{99}, t_{1lg}, and especially T_{min} may be only approximate. Moreover, the use of multiple parameters often does not allow the definitive assessment of AME, because of conflicting values of T_{99}, B_{min}, and t_{1lg}. A comprehensive analysis of these and other AME parameters is presented elsewhere (8).

In view of the above-mentioned considerations, we have recommended that AME duration (T_E) can be used as an integral characteristic of bacterial growth curves (S. M. Navashin, A. A. Firsov, V. M. Chernykh, and S. M. Kuznetsova, Proc. 14th Int. Congr. Chemother., p. 722–723, 1985). This parameter is determined by time from antibiotic injection into a dynamic model to the moment when bacterial heat production (as determined by microcalorimetry), inoculum opacity (as determined by turbidimetry), or CFU count (as determined by the conventional counting procedure) again reaches the initial level.

In contrast to the parameters T_{99}, T_{min}, and t_{1lg}, T_E can be considered a characteristic not only of the curve describing the change in microbial count but also of AME itself. This is due to the fact that T_E reflects the shift between the growth curve in the absence of an antibiotic and that of microbial regrowth when the drug concentration falls in the dynamic model. On the other hand, T_E may not reflect the intensity of AME (I_E). For this reason, another integral index of AME, I_E, was introduced and studied in the present work. In doing so, we considered the possibility of establishing reasonable effect-concentration or, strictly speaking, AME parameter-pharmacokinetic parameter relationships as the main criteria for the applicability of one or another AME parameter.

The alternative means for expressing AME were analyzed in a microcalorimetric study of the kinetics of the AME of sisomicin on *Klebsiella pneumoniae* 5056, *Pseudomonas aeruginosa* 58, and *Escherichia coli* 93 in a dynamic model (14). Sisomicin pharmacokinetic profiles (one-compartment model with first-order absorption) observed after intramuscular administration of the drug to humans were simulated and reflect the individual variability of levels in blood typical of aminoglycosides.

MATERIALS AND METHODS

The kinetics of the AME of sisomicin on *P. aeruginosa* 58, *E. coli* 93, and *K. pneumoniae* 5056 were studied by using a dynamic model (Fig. 1) described earlier (14). The MICs for these strains as determined by the method of double serial dilutions in broth (pH 7.2 to 7.3) were 0.25, 0.25, and 0.5 μg/ml, respectively.

Vessel 0 in this model is a glass tube with two syringe needles fitted with silicone pipes connecting vessel 0 to the vessel with fresh nutrient broth and vessel 1 containing bacterial cells. The fresh broth is supplied to vessel 0 by pump P_1. From vessel 0 the broth flows under pressure to vessel 1, which is also a glass tube but with four syringe

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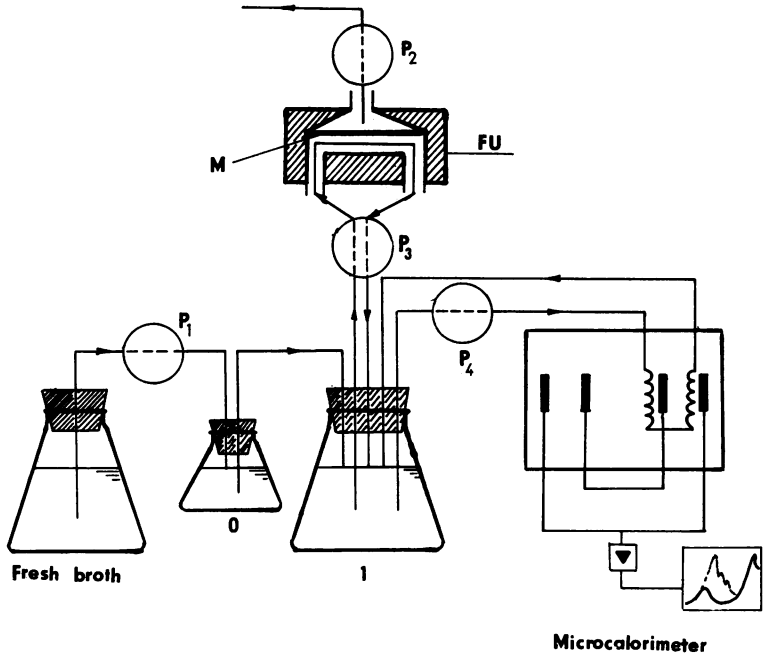

FIG. 1. Design of the dynamic system. FU is the filtration unit; M is a membrane filter; P_1, P_2, P_3, and P_4 are peristaltic pumps. The arrows indicate flow directions. Shown on the right is the registration unit.

needles. From vessel 1 the antibiotic solution flows to the microcalorimeter by pump P_4 and to the filtration unit by pump P_3. The body of the unit is made of organic glass. To prevent membrane-pore blocking, the medium containing bacterial cells and drug molecules from vessel 1 is continuously run over its surface with pump P_4. Pump P_2 provides forced removal of the antibiotic solution through the filtration membrane at the same flow rate as that of pump P_1.

The sisomicin concentration-time profiles were simulated in the central compartment (1 in Fig. 1) containing microbial cells. The values of the elimination rate constant (0.0056 min^{-1}) and the first-order absorption rate constant (0.1008 min^{-1}) were adopted from the literature on sisomicin human pharmacokinetics (compiled data [14]). The values of the simulated drug maximum concentrations were 0.55, 1.1, 2.2, 4.4, and 8.8 μg/ml. The values of the volumes of the central compartment (89.3 ml) and the subcompartment (4.9 ml) of the dynamic model and the flow rate (0.5 ml/min), as well as the drug amounts administered into the subcompartment (58.2, 116.4, 232.9, 465.7, and 931.4 μg, respectively), were calculated as described earlier (14). The simulated pharmacokinetic profiles are outlined in Fig. 2.

After the system was filled with sterile broth and incubated at 37°C, 0.05 ml of an 18-h culture was added. The onset of the logarithmic growth phase was detected by the heat production rate (dQ/dt). When the rates reached 25 μW/ml ($4 \cdot 10^5$ CFU/ml) for *P. aeruginosa*, 62 μW/ml ($1.9 \cdot 10^6$ CFU/ml) for *E. coli* 62, and 50 μW/ml ($2.5 \cdot 10^6$ CFU/ml) for *K. pneumoniae* 5056, sisomicin was added to vessel 0, and a peristaltic pump was started to maintain the given regimen of drug level change in the main compartment. Additional pumps supplied medium from vessel 0 to the filtration unit and a microcalorimeter (4, 14).

Microbial counts were determined with an LKB model 2277-202 microcalorimeter (LKB, Stockholm, Sweden) operated in the flow mode at 37°C in the registration range of 0 to 300 μW. Medium was supplied from vessel 1 at a rate of 20 ml/h with pump P_4 (Microperpex 2132; LKB).The effective volume of the registration cylinder was 0.55 ml. Calibration procedures and the operation sequence have been described in detail elsewhere (3, 15, 21).

More detailed information on the sensitivity, specificity, and reproducibility of the microcalorimetric technique was published earlier (1, 4, 8, 11).

To correlate the dQ/dt with bacterial cell concentration (CFU per milliliter), samples of the cell suspensions of *P. aeruginosa* 58, *E. coli* 93, and *K. pneumoniae* 5056 were periodically taken from vessel 1. The CFU were then

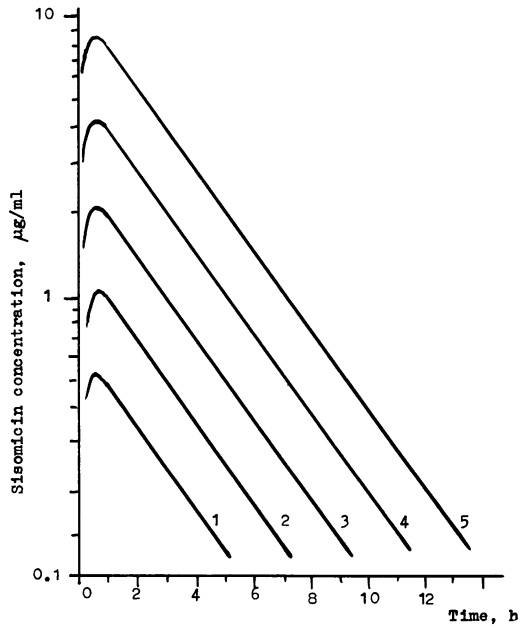

FIG. 2. Sisomicin pharmacokinetic profiles simulated in the dynamic model. Maximum concentrations were as follows: 1, 0.55 μg/ml; 2, 1.1 μg/ml; 3, 2.2 μg/ml; 4, 4.4 μg/ml; and 5, 8.8 μg/ml.

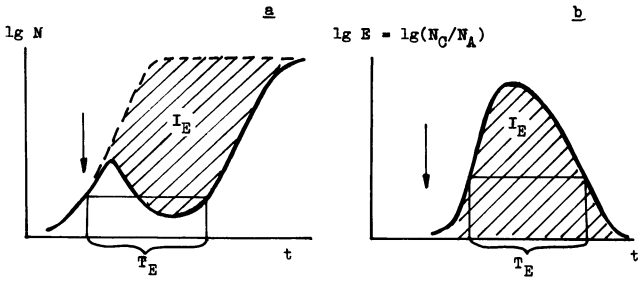

FIG. 3. Theoretical analysis of AME kinetic curves in dynamic systems with the registration of CFU number (b). (a) Hypothetical curves of microbial growth in the presence of an antibiotic (solid line) and the control (dashed line). The arrow indicates the moment of drug administration.

counted on a Bio Tran II model C III automatic counter (New Brunswick Scientific Co., Inc., Edison, N.J.).

Correlation analysis of the T_E values for different sections of the dQ/dt-versus-time curves, as well as of the T_E-versus-I_E and CFU-per-milliliter-versus-dQ/dt relations, was performed on an SM-4 computer (CORRE program).

RESULTS

Theoretical: integral characterization of AME kinetic curves. Parameterization of AME kinetic curves is closely associated with the way in which AME is expressed. Such expression must rely on a comparison of the curves of microbial count change in the presence [$N_A(t)$] and absence [$N_C(t)$] of an antibiotic, where A is the antibiotic, C is the control, and t is time. AME (E) may be expressed as the reciprocal of the ratio N_A/N_C, that is,

$$E(t) = N_C(t)/N_A(t) \quad (1)$$

or as the difference $[-(N_A - N_C)]$ at some instant:

$$E'(t) = N_C(t) - N_A(t) \quad (2)$$

Each of the above approaches gives a net estimate for AME, since the continuous drift of control growth is considered. The former approach is more convenient when the index of microbial count employed varies over a wide range (e.g., when CFU are counted), while the latter approach is used when this range is relatively narrow (e.g., when turbidimetric, microcalorimetric, and other techniques are used).

One of the integral characteristics of AME is T_E. This value is a measure of the regrowth-curve shift in the presence of an antibiotic relative to the growth curve in the absence of an antibiotic. As is seen from the log $E(t) = \log_{10}[N_C(t)/N_A(t)]$-versus-$t$ plot reflecting the kinetics of AME increase and disappearance (Fig. 3b), T_E characterizes the effective duration of AME. I_E may be quantified by measuring the area under the AME kinetic curve (Fig. 3b). This can be the log $E(t)$ curve if AME is determined in terms of equation 1 and the $E'(t)$ curve in the case of equation 2. This means for assessing I_E is especially appropriate since the parameter I_E corresponds with the area under the antibiotic concentration-versus-time curve (AUC) as an integral pharmacokinetic parameter to ultimately reveal the concentration dependence of the AME.

The preliminary calculation of log $E(t)$ and $E'(t)$ is not necessary for evaluating I_E, since I_E is given by the area between the growth curves in the absence and presence of a drug (Fig. 3a). Therefore, both integral parameters, T_E and I_E, can be determined from the log $N(t)$ or $N(t)$ plot.

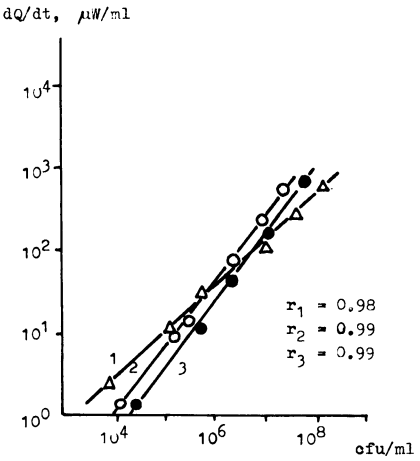

FIG. 4. Relationship between dQ/dt and the count of microbial cells. 1, *P. aeruginosa* 58; 2, *E. coli* 93; and 3, *K. pneumoniae* 5056. Cell concentrations (△, ○, and ●) were evaluated by counting CFU; r is the correlation factor.

In revealing the concentration dependence of AME, it is better to compare I_E and T_E to pharmacokinetic parameters, such as AUC, only within the AUC range in which regrowth is observed and I_E and T_E have finite values. With bactericidal AME, such comparison and quantitative correlation of I_E or T_E and AUC become senseless.

Experimental. Figure 4 presents relationships between the dQ/dt and cell counts (CFU per milliliter) for the three strains studied in the dynamic model without antibiotic. The relationships are linear over the range of 10^4 to 10^8 CFU/ml, although each regression line has a different slope. Correlation coefficients for *P. aeruginosa*, *E. coli*, and *K. pneumoniae* proved to be high. This points to the reliability of the indirect registration of microbial count by the microcalorimetric technique. Since dQ/dt-versus-CFU-per-milliliter dependence is linear, one can easily calculate CFU per milliliter from dQ/dt.

Figure 5 presents thermograms for the time courses of counts of *P. aeruginosa*, *E. coli*, and *K. pneumoniae* in the dynamic model in the absence and presence of sisomicin. The pharmacokinetic profiles observed after a single intramuscular drug administration to humans were simulated in the latter case. Sisomicin decreased the dQ/dt as a result of microbial growth inhibition and killing, while in the absence of the drug the rate increased steadily. As the sisomicin level in the central compartment decreased, the dQ/dt began to increase, again reflecting the resumption of cell proliferation. The regrowth curves and those of microbial growth in the absence of the antibiotic are similar. Therefore, microbial generation times remained unchanged after incubation in a sisomicin-containing medium.

Figure 5 also shows that the time from drug injection to growth resumption increased with antibiotic concentration and respective AUCs, with the regrowth curves being shifted to the right. T_E values were calculated to reveal the concentration dependence of AME.

The T_E-versus-AUC curves for sisomicin are shown in Fig. 6. They demonstrate that T_E increased with AUC, and they infer that, in the AUC range of 13 to 26 μg · h/ml corresponding to therapeutic doses of sisomicin, T_E decreased in the order *K. pneumoniae* > > *E. coli* > *P. aeruginosa*.

Thus, the use of the T_E parameter helped to correlate drug

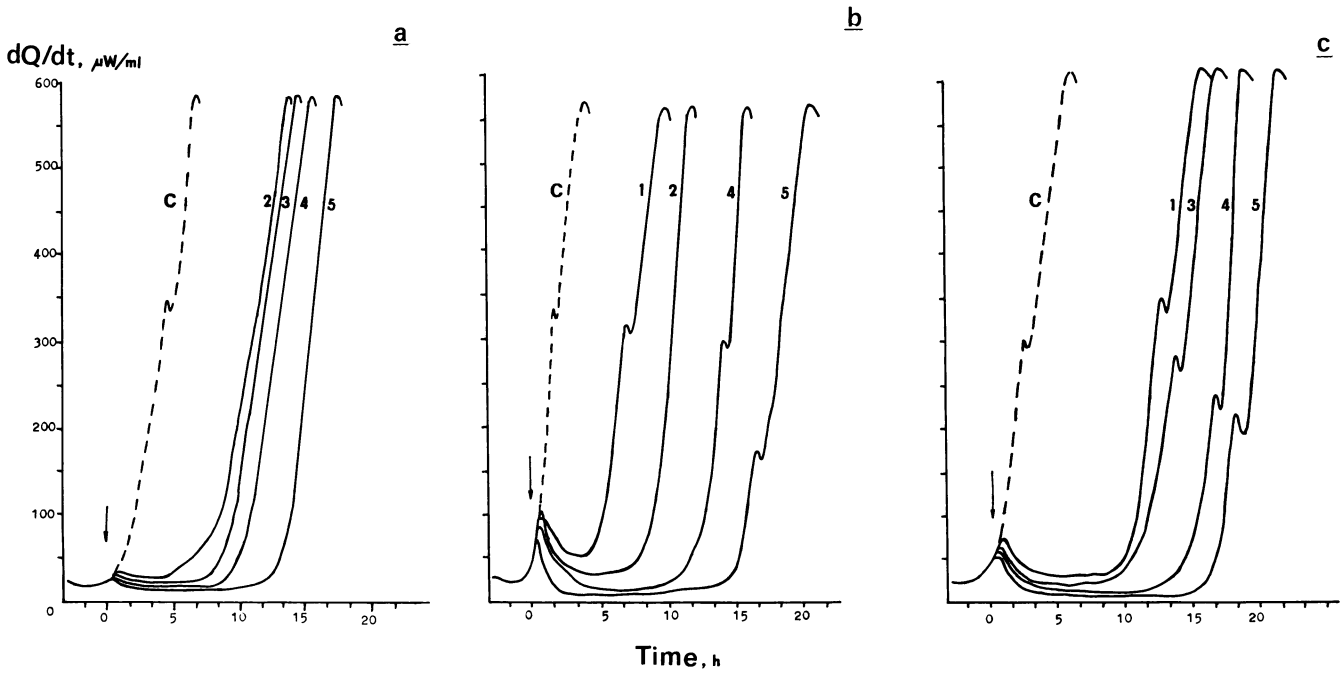

FIG. 5. Kinetics of dQ/dt for *P. aeruginosa* 58 (a), *E. coli* 93 (b), and *K. pneumoniae* 5056 (c) during simulation of different sisomicin pharmacokinetic profiles. Dashed lines represent thermograms in the absence of the antibiotic (C, control). Numbers at solid lines correspond to different pharmacokinetic profiles shown in Fig. 2. The arrows indicate the moment of drug administration.

effect and AUC and to lead to the conclusion that differences in drug AME occur with different tested strains. The results of assessing sisomicin AME by simulating pharmacokinetic profiles in a dynamic model are inconsistent with preliminary data obtained by evaluating the MIC. In contrast to T_E, MICs for the strains studied were similar.

There is a strong correlation between T_E values corresponding to the heat output rates at the moment of drug injection (25 to 62 μW) and the values observed at higher rates (200 μW), corresponding to the central fragment of the logarithmic growth phase. This correlation allows T_E to be estimated at any bacterial cell concentration or heat output rate. Thus, the sensitivity of the procedure for counting microorganisms is not a factor in estimating T_E, and T_E can be also evaluated by using the relatively insensitive turbidimetric technique.

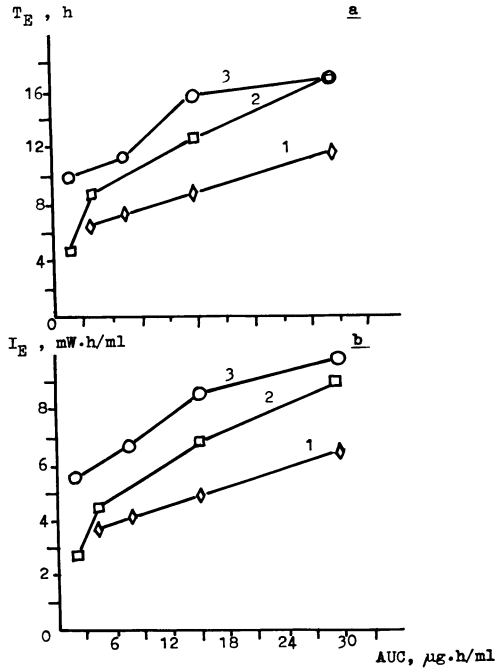

FIG. 6. Dependence of T_E and I_E on the sisomicin AUC. 1, *P. aeruginosa* 58; 2, *E. coli* 93; 3, *K. pneumoniae* 5056.

Regarding the assessment of T_E from microcalorimetric data, the CFU count correlates with the dQ/dt up to the maximal I_E value, as given by the area between the heat-output-rate-versus-time curves in the experiment and in the control up to the maximal rate (Fig. 6). I_E actually characterizes a fragment of the area under the $E'(t)$ or $N_C(t) - N_A(t)$ curve.

Figure 6 shows the dependence of I_E on AUC. As seen in Fig. 6b, I_E increases with AUC and the shape of I_E versus AUC is similar to that of T_E versus AUC. This is consistent with the close correlation observed between I_E and T_E (r = 0.97).

The monitoring of the dQ/dt up to maximum values is apparently not necessary for correlating I_E to AUC. Clearly, the horizontal section of the curves of heat production kinetics must be chosen so that the corresponding dQ/dt is higher than the maximum value measured before kinetic curves reach their minimum.

DISCUSSION

The theoretical analysis of AME kinetics performed in the present work suggests that AME is to be characterized by the integral parameters of T_E and I_E. The former is determined by the microbial-regrowth-curve shift in the presence of an antibiotic relative to the growth curve in the absence of drug. The latter is given by the area between these curves. Surely, the integral estimation of AME with the parameters

T_E and I_E by no means excludes the possibility of detailed kinetic analysis of curves such as the one depicted in Fig. 3b. In particular, the time course of AME development rate can be analyzed.

The fundamental difference between the parameters T_E and I_E and those proposed earlier (19) is that the former characterize AME itself rather than the curve of microbial growth or the corresponding variation of optical density or dQ/dt. The parameters T_E and I_E provide an unequivocal estimate for AME, which is rather difficult, if not impossible, with the multiparametric treatment of growth curves. Also, the tendency to use integral estimates of microbial growth curves in the presence of an antibiotic can be distinctly traced in the recent literature. Attempts have been made to characterize these curves by the number of CFU at the end of the drug dosage interval (at the moment of the next drug injection) or by the ratio of this number to that at the moment of antibiotic administration (5, 12, 13, 16, 17, 22–24). In other words, the vertical section of kinetic curves (by time) is usually considered. At first glance, such an approach seems reasonable since it directly considers antibiotic dosage regimens used clinically, but in many cases it does not apply.

Thus, if the kinetic curves compared are in different phases at a given moment or if at least one of them is in the stationary growth phase, a quantitative comparison of microbial counts at the moment of the vertical section is not possible. In addition, this approach as well as the parameters T_{99}, B_{min}, T_{min} and t_{1lg} can be used, and even then with reservations, only as they are useful to characterize the curves of microbial growth inhibition and resumption in the presence of an antibiotic, but they are not useful to assess AME.

It should be noted that unlike for the T_E or I_E parameter, multiple-parametric evaluation of the above-mentioned curves often does not allow one to get an ultimate conclusion about AME. Thus, although the action of cefotetan against one of two *K. pneumoniae* strains previously studied (20) was characterized by a slower decrease in the CFU count (T_{99} = 0.9 h) and a higher minimum concentration (B_{min} = 0.044%), the time for reaching the minimum CFU number (T_{min} = 4 h) and the regrowth rate (t_{1lg} = 3.8 h) were smaller than the respective parameters for the other strain (T_{99} = 0.44 h, B_{min} = 0.001%, T_{min} = 8 h, t_{1lg} = 1.0 h) (20). It therefore remains unclear which of the strains is more susceptible to the cephalosporin.

The possibility of using the parameters T_E and I_E for revealing the concentration dependence of AME was experimentally verified in the present work with a microcalorimetric study of the AME of sisomicin on gram-negative microorganisms. The drug pharmacokinetic profiles observed after intramuscular administration to humans were simulated with a dynamic model. T_E was calculated directly from the kinetic curves of the dQ/dt in microorganisms. I_E was assessed by using the area between the kinetic curves of the dQ/dt in the presence and absence of the antibiotic. Since the shift of the dQ/dt-change curve in the presence of sisomicin relative to the control curve was virtually independent of the dQ/dt value (i.e., of the level of the horizontal section of the kinetic curves), T_E values measured at different dQ/dt were equal. Clear correlation has been found to exist between T_E and I_E. T_E and I_E increased steadily with sisomicin concentration. The analysis of the dependence of T_E on the AUC has allowed unequivocal conclusions as to the antibiotic AME on the strains tested. Moreover, the T_E values are practically insensitive to the techniques used for bacterial cell concentration recording. The T_E estimates for sisomicin and *E. coli* A 20363 obtained with a counting procedure and turbidimetric and microcalorimetric techniques were virtually the same despite different sensitivities of the methods (Navashin et al., 14th ICC). Recently, differences in the action of netilmicin on *P. aeruginosa*, *E. coli*, and *K. pneumoniae* have been revealed by using a similar approach, different pharmacokinetic profiles of the antibiotic being simulated in a dynamic model (2).

Some perspectives on the application of the T_E or I_E parameter were shown in comparative studies of the AME of a cephalosporin (A. A. Firsov, A. D. Nazarov, V. M Chernykh, P. S. Navashin, and L. N. Samoylova, 3rd Eur. Congr. Clin. Microbiol., abstr. no. 275, 1987), the AME of sisomicin on various bacterial strains (8), the AME of various ampicillin-sulbactam combinations (9), the influence of different routes (8, 9) or regimens of antibiotic administration (7), and, of course, evaluation of AME-concentration dependences (6; A. A. Firsov, Proc. 3rd Eur. Congr. Biopharmaceut. Pharmacokinet., p. 269–276, 1988). Thus, the T_E or I_E estimation appears to be a promising approach in the evaluation of AME.

In conclusion, the AME of sisomicin on the strains tested did not correlate with the respective MICs. Others also have pointed out the unreliability of the MIC as a predictor of AME (17, 18, 24). This again indicates the importance of considering pharmacokinetic factors in assessing the AME of antibiotics.

ACKNOWLEDGMENT

We thank S. H. Zinner for his useful advice and remarks in preparing the manuscript.

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